Mismatch repair protein hMSH2 in primary drug resistance in vitro human malignant gliomas

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Object. The mismatch repair (MMR) system has previously been implicated in acquired chemoresistance in malignant gliomas in humans. Its impact on the primary chemoresistance in glioblastoma multiforme (GBM) has not been determined in detail, however.

Methods. The authors investigated the expression of both the MMR genes (hMSH2, hMLH1, hPMS1, hPMS2, and hMSH6) at the transcriptional level through reverse transcription–polymerase chain reaction and the hMSH2 protein through Western blot and immunohistochemical analysis of tumor tissue and primary cell cultures of 25 in vitro human de novo GBMs without prior experimental treatment. Results of these analyses were compared with data on in vitro chemoresistance to nine drugs that are in general use in glioma therapy.

All MMR genes were expressed in the GBMs, with no significant difference among the individual tumors except in one respect; that is, GBMs showed either relatively high levels or barely detectable levels of hMSH2 messenger (m)RNA and protein expression. All multiresistant tumors demonstrated high hMSH2 expression, and all but two of the sensitive tumors exhibited low hMSH2 mRNA levels.

Conclusions. Analysis of the data indicates that functional alterations of the MMR system are involved in the primary drug resistance in in vitro human malignant gliomas. Analysis of hMSH2 expression might therefore predict therapeutic responses in humans with GBMs.

KEY WORDS • glioblastoma multiforme • DNA mismatch repair • chemotherapy • drug resistance

Abbreviations used in this paper: ACNU = 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea; BCNU = 1,3-bis(2-chloroethyl)-1-nitrosourea; GBM = glioblastoma multiforme; HNPCC = hereditary nonpolyposis colon carcinoma; MMR = mismatch repair; mRNA = messenger RNA; RT-PCR = reverse transcription–polymerase chain reaction.
with methylthiotetrazole (1 mg/ml; Sigma-Aldrich) and was added teniposide (Bristol-Myers Squibb, New York, NY), 4

**TABLE 1**

In vitro chemosensitivity test results for 25 GBMs*

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* R = resistant; SEN = sensitive; — = not determined.

**Reverse Transcription–Polymerase Chain Reaction**

The process of RT-PCR was performed according to the methods outlined by Wei, et al. Briefly, complementary DNA synthesis was conducted with 250 ng of total RNA by using Moloney murine leukemia virus reverse transcriptase (Gibco BRL) and random hexanucleotides (Boehringer, Mannheim, Germany) in a final volume of 20 µl. The PCR amplifications were performed using 5 µl of the first-strand reaction product in three mixtures; one contained 125 pmol HmHS2 and 20 pmol HMLH1 primers; another contained 25 pmol hPMS1, 50 pmol hPMS2, and 30 pmol hMMH2 primers; and a third mixture contained 15 µM β-actin primers. Each of the three reaction mixtures containing 0.2 µM each deoxynucleoside triphosphates, 50 mM KCl, 1 mM Tris-Cl (pH 8.8, 25˚C), 1.5 mM MgCl2, 1 mM dithiothreitol, and 2.5 U Taq polymerase (Qiagen) in a final volume of 50 µl was amplified by PCR (first cycle at 95˚C for 5 minutes; 29 cycles at 95˚C for 1 minute; 59˚C for 1 minute; 72˚C for 1.5 minutes; and a final cycle at 72˚C for 5 minutes). The PCR products were applied to nondenaturing polyacrylamide gels and visualized by silver staining.

**Western Blot Analysis**

Ten micrograms of protein was loaded into 10% polyacrylamide/sodium dodecyl sulfate gels and separated at a constant voltage of 90 V. After electrophoresis, proteins were transferred to Immobilon PVDF membranes (Millipore, Billerica, MA) at 200 mA for 60 minutes. Membranes were blocked for 1 hour by using 5% skim milk powder in Tris-buffered saline with Tween-20 and incubated overnight at 4˚C with monoclonal antibodies against hMSH2 (clone FE11; Oncogene Research Products, Cambridge, MA) and p53 (clone DO-7; DAKO, Hamburg, Germany). The membranes were incubated for 1 hour at room temperature with horseradish pero-
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d—conjugated antibodies diluted to a concentration of 1:2000 in Tris-buffered saline with Tween-20 before immunodetection with LumiGLO reagent (Cell Signalling Technology, Beverly, MA). The immunoreaction was detected by exposing the membrane to X-OMAT MA-film (Kodak, Paris, France).

Immunohistochemical Studies

Tumor biopsy samples from 12 GBMs were also investigated through immunohistochemical means by using an indirect immunoperoxidase reaction. Briefly, sections of paraffin-embedded tumor tissues were cut to 6 μm and incubated for 1 hour at 37°C with monoclonal antibodies against hMSH2 (clone FE11, dilution 1:200) after microwave pretreatment at 95°C for 30 minutes in citrate buffer, pH 6.0. For negative controls the first antibody was omitted. Biotinylated horse anti—mouse immunoglobulin G (50 μl in 10 ml phosphate-buffered saline, 30 minutes) served as a secondary antibody, and avidin-biotin-horseradish peroxidase was used as the detection system. Immunoreactivity was visualized with 3,3′-diaminobenzidine tetrahydrochloride (final concentration 5 mg/ml in 0.05 M Tris buffer, pH 7.6, 15 minutes). Tissue sections were counterstained with Mayer hemalum stain. Immunoreactivity was estimated qualitatively by using a four-point grading scale (+ + +, strong immunoreactivity; ++, moderate immunoreactivity; +, weak immunoreactivity; −, no immunoreactivity).

Statistical Analysis

All gels were scanned and subjected to densitometric analysis by using the NIH Image software package (National Institutes of Health, Bethesda, MD). Densitometric values of RT-PCR with β-actin were compared with corresponding reference values obtained from lymphocytes.19 Differences in the frequency distribution between two or more samples were analyzed using the chi-square test. The differences in expression levels between tumor groups were calculated using the nonparametric U-test.

Results

In Vitro Chemosensitivity Testing

Primary cell cultures of 25 primary de novo GBMs were tested for in vitro chemosensitivity to ACNU, BCNU, cytarabine, cisplatin, mitoxantrone, methotrexate, vincristine, teniposide, and Taxol. Tumor in vitro chemoresistance to one of the agents was defined as the presence of more than 50% viable tumor cells after drug exposure. Based on this definition, all tumors showed resistance to the nitrosourea compounds ACNU and BCNU. Only one tumor each exhibited sensitivity to cisplatin and methotrexate. In contrast, 12 (48%) of 25 GBMs were resistant to cytarabine, 18 (72%) of 25 to mitoxantrone, 10 (40%) of 25 to vincristine, 14 (56%) of 25 to teniposide, and 10 (45%) of 22 to Taxol (Table 1). Nine (36%) of 25 GBMs were resistant to all drugs tested—these tumors were defined as “multiresistant”—and one tumor showed sensitivity to one drug only. Two tumors in the multiresistant group were resistant to lomustine and Temodal (data not shown). In contrast, 15 tumors (60%) exhibited sensitivity to three or more drugs; these tumors were defined as “sensitive.”

Detection of mRNA Expression of MMR Genes by RT-PCR

Results of RT-PCR as an indicator of the expression of MMR genes revealed no difference in the intensities of the specific PCR fragments of the mRNAs of hMLH1 (215 bp), hPMS1 (174 bp), hPMS2 (359 bp), hMSH6 (alias GTBP, 288 bp), and β-actin (180 bp), which served as a control in comparisons between individual tumors (Fig. 1a). In contrast, there were significant differences in the intensities of the PCR fragment generated from hMSH2 mRNA (429 bp; Fig. 1a). Twelve tumors showed an intense PCR fragment and 13 GBMs exhibited a weak or almost absent PCR fragment. According to results of densitometry, 12 tumors demonstrated hMSH2/β-actin values greater than 0.82, whereas 13 tumors exhibited values of less than 0.56 (Fig. 2). Interestingly, all multiresistant tumors but only two sensitive tumors demonstrated strong hMSH2 bands. The mean densitometric value in multiresistant tumors was 1.03 (range 0.82–1.29) and in sensitive tumors it was 0.41 (range 0.14–1.1; p < 0.01, U-test).

Detection of hMSH2 Protein Expression by Western Blot and Immunohistochemical Analyses

There was a close correlation between the hMSH2 mRNA expression detected on RT-PCR and on Western blot analysis with antibodies directed against the hMSH2 protein (p < 0.01, chi-square test; Fig. 3). Twelve tumor samples were also subjected to immunohistochemical analysis by using anti-hMSH2 antibodies (Fig. 4). We also found a close correlation between data obtained by Western blot analysis on tumor cell cultures and immunohistochemical studies of tumor samples in vivo (p < 0.01, Fisher exact test). Immunostaining was heterogeneous within individual tumors, but lesions with high hMSH2 protein levels on Western blot analysis demonstrated positive nuclear reaction in approximately 60 to 70% of tumor cells; the gliomas with low levels of hMSH2 protein on Western blot analysis showed nuclear immunostaining in only approximately 30% of tumor cells. Only one GBM demonstrated high levels of hMSH2 protein on Western blot analysis and only approximately 40% immunoreactive cells on immunohistochemical testing. Note, however, that this sample was derived from the infiltration zone and/or edges of the tumor.

Discussion

Despite considerable advancements in the clinical management of malignant gliomas, their prognosis remains poor and only a few patients benefit from aggressive multimodal treatments including chemotherapy.7 So far, the mechanisms leading to extreme chemoresistance in gliomas are poorly understood. At least two components of drug resistance have been described in malignant gliomas: acquired chemoresistance after initially successful therapy and primary chemoresistance. The aim of the present study was to investigate the functional role of the MMR system in the primary drug resistance of in vitro human GBMs. Therefore, the acute cytotoxicity of a panel of drugs currently used in standard chemotherapy protocols was determined using an in vitro chemosensitivity test in de novo GBMs. Using this approach we found two groups of GBMs: one subgroup of multiresistant gliomas demonstrating no response to any of the drugs and another subgroup of lesions sensitive to three or more drugs. We did not study the therapeutic response in individual patients, but instead used an in vitro chemosensitivity test for several reasons. Treatment of malignant gliomas requires multimodal therapies including radical resection and radiotherapy. Response rates and survival data in patients are the result of all these combined treatments. The clinical effects of adjuvant che-
motherapy can therefore only be evaluated in large clinical studies. Use of the in vitro chemosensitivity test, however, provided us with quantitative data about the response of individual GBMs to different cytotoxic drugs. Although its predictive value for successful therapy is poor, it predicts tumor resistance exactly. It has been shown in retrospective studies that the assay correctly predicts clinical response to chemotherapeutic agents in only 50 to 70% of patients; clinical resistance, however, has been predicted in approximately 100% of patients.9,16

We decided to investigate only de novo GBMs without prior treatment to exclude functional defects due to genetic alterations of the TP53 gene. In human malignant gliomas genetic alterations of TP53 are mainly restricted to secondary GBMs, whereas de novo GBMs rarely exhibit TP53 mutations.18 In accordance with this view we detected only one tumor with a TP53 mutation in the present study (data not shown). In conditions without (chemotherapy) treatment we detected the expression of all MMR genes (hMSH2, hMLH1, hPMS1, hPMS2, and hMSH6) at the transcriptional level. All but hMSH2 showed no significant difference between individual tumors. The expression of

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**Fig. 1.** Gel blots obtained by performing RT-PCR. All resistant (res) tumors exhibited high levels of hMSH2 expression, and all but one (Lane 13) of the sensitive (sen) tumors expressed low levels of hMSH2 (a). No difference in the expression of hMLH1 and β-actin (a) or that of hPMS1, hPMS2, and hMSH6 (b) could be detected in comparisons between individual tumors (silver-stained polyacrylamide gels).

**Fig. 2.** Dot plot demonstrating densitometric values of RT-PCR results for hMSH2 and β-actin expression. The quotient of the hMSH2 and β-actin values was compared between resistant and sensitive tumors.
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hMSH2 was different, however, leading us to define at least two groups of GBMs: one subgroup with relatively high expression levels and another subgroup with barely detectable levels of expression. Interestingly, we found a close correlation between in vitro chemosensitivity results and hMSH2 expression patterns. All multiresistant tumors showed high hMSH2 expression, and all but two of the tumors sensitive to three or more drugs exhibited low hMSH2 expression. There was a close correlation between the protein expression on Western blot analysis and that on immunohistochemical testing of corresponding tumor samples, indicating that the constitutive expression of the hMSH2 protein is regulated at the transcriptional level.

Analysis of our data indicates that hMSH2 plays a role in the primary drug resistance of in vitro human malignant gliomas and that high hMSH2 expression might be a predictor of primary chemoresistance. These results contrast with chemosensitivity data obtained in studies of sporadic and familial cancers belonging to the HNPCC spectrum of tumors. In these tumors the loss of hMSH2 due to mutations of the hMSH2 gene is correlated with resistance to chemotherapeutic agents. Mutations of MMR genes are the hallmark of the familial cancer syndrome HNPCC. They are, however, absent in sporadic malignant gliomas. It has been speculated that functional downregulation rather than genetic alterations of MMR genes in glioma cells could contribute to the acquired form of chemoresistance. Experimental data have demonstrated that hMSH2 was downregulated in a human GBM xenograft that had been treated repeatedly with procarbazine to induce chemoresistance. Note, however, that MMR deficiency can arise not only through mutation or transcriptional silencing of an MMR gene but also as a result of the overexpression of MMR proteins.

These experimental data cannot be directly compared with the results of the present study. Here, we investigated the constitutive expression without previous drug treatment in primary human GBM cell cultures. The results of our in vitro study seem to be representative of the in situ context because the immunohistochemical analysis of hMSH2 protein expression in the GBM biopsy samples closely correlated with the primary cell culture data. Analysis of our data indicates that the role of MMR genes in untreated human GBMs is different from the situation in tumors of the HNPCC spectrum and also distinct from experimental models of acquired resistance in gliomas.

What, in fact, is the functional role of MMR genes in malignant gliomas prior to therapy? There are few data about MMR genes in gliomas. Wei and coworkers reported that the low expression of MMR genes measured using the multiplex RT-PCR approach, which was adopted by us, frequently occurs in gliomas in humans. In their study approximately half of the gliomas showed a significant reduction in hMSH2, which is in accordance with our data (52%). Constitutive defects in the MMR system should lead to microsatellite instability, which is, however, absolutely rare in malignant gliomas and occurs in only 0 to 3% of GBMs. One would expect a higher rate of microsatellite instability in malignant gliomas if MMR downregulation were to have functional effects. Clearly, this is not the case. Therefore, low expression levels of hMSH2 in human gliomas is not equivalent to the loss of expression due to mutations in tumors of the HNPCC spectrum. More likely, these low levels could indicate a functional status of low repair capacity. The high hMSH2 expression in the majority of the resistant tumors in our study could correspond to an upregulation of the MMR system, probably resulting in an enhanced repair capacity. Consequently, tumors with high levels of hMSH2 are a priori more resistant to chemotherapy, a point of view that is corroborated by data from other studies in which similar findings have been reported for other tumors.

Conclusions

We demonstrated that GBMs with high expression of the MMR protein hMSH2 are multiresistant by using an acute

FIG. 3. Gel blots of six tumors that tested resistant and nine tumors that tested sensitive, showing high hMSH2 protein levels in all resistant tumors and only two sensitive tumors. The protein kinase B (PKB) was used as a loading control.

FIG. 4. Photomicrographs demonstrating representative immunohistochemical study results. One tumor with high protein expression detected on Western blot analysis revealed strong nuclear immunoreactivity (left), whereas another tumor with low protein levels on Western blot analysis showed barely detectable hMSH2 immunoreactivity (right). Original magnification × 20.
cytotoxicity assay. Analysis of these data indicates that functional alterations of the MMR system are involved in primary drug resistance of malignant gliomas and might therefore be valuable at least for the prediction of resistant tumors. Furthermore, the functional analysis of repair systems could identify predictive factors and thus reveal novel treatment approaches.

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References


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